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IN VITRO PROPAGATION OF PELECYPHORA ASELLIFORMIS EHRENBERG AND P. STROBILIFORMIS WERDERMANN (CACTACEAE)

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Summary

Development of efficient in vitro propagation systems for Pelecyphora aselliformis Ehrenberg and P. strobiliformis Werdermann, two endangered Mexican species of cacti, are described. Multiple shoot formation from areoles of in vitro-germinated plantlets was achieved in two types of explants (apical and transversal) cultured in Murashige and Skoog (MS) basal media supplemented with 30 or 50 g l^{-1} sucrose, 10 g l^{-1} agar and various treatments with cytokinins. Shoot production in these proliferation media was evaluated after one (60 d) and three (180 d) culture cycles. In P. aselliformis 13.7 shoots per explant were produced after the first cycle using apical explants in medium with 8.8 μM 6-benzylaminopurine (BA) and 30 g l^{-1} sucrose. In P. strobiliformis the highest proliferation rate (12.4 shoots per explant) was reached using 8.8 μM BA and 50 g l^{-1} sucrose with shoot transverse segments as explants. After the third proliferation cycle, 128.1 and 136.3 shoots per explant were obtained in P. aselliformis and P. strobiliformis, respectively. The shoots were elongated in MS basal medium with 3 g l^{-1} activated charcoal and rooted in MS basal media with indoleacetic acid (2.85 or 5.71 μM) or indolebutyric acid (2.46 or 4.90 μM). On average, rooting efficiency was 89% for P. aselliformis and 87% for P. strobiliformis. The survival frequency of the plants once transferred to soil was on average 88%.

Key words: areole activation; cytokinin; micropropagation; tissue culture.

Introduction

Mexico has the richest diversity of cacti worldwide. Unfortunately, about 25% of all cacti species are listed as endangered because of habitat loss and illegal collecting from the wild, making this family one of the most likely to become extinct in the plant kingdom (Hubstenberger et al., 1992). The genus Pelecyphora includes two of the more rare and endangered species of Mexican cacti. P. aselliformis is a small plant with a solitary and cespitose stem, 1–3 cm high and 2–5 cm in diameter, in habitat lying almost flat with the ground. This species is endemic to San Luis Potosí State and is only known from a few localities. P. strobiliformis is a plant with a solitary and occasionally cespitose stem, about 3.5 cm high and 8 cm in diameter, distributed in the States of San Luis Potosí, Tamaulipas and Nuevo León. Both species are highly prized by collectors. Some populations have been virtually eliminated and the remaining ones show definite signs of illegal collecting, so the species must be considered threatened (Glass, 1998). The production of seeds and germination rate in these species are low, and their growth is extremely slow. For these reasons it is difficult to recover endangered populations through natural procedures, and conventional propagation methods are often inadequate for those cacti that exhibit low germination rates and low or no lateral branching. Therefore, it is necessary to safeguard these species and to improve, in any possible way, the propagation techniques.

During the past few years, plant tissue culture has emerged as a powerful tool for the effective propagation of threatened cacti (Hubstenberger et al., 1992). These techniques have the potential to produce a great number of plants in a short time and in minimal space, and their success has already been demonstrated for several endangered cacti species such as Mammillaria san-angelensis (Martínez-Vázquez and Rubluo, 1989), Aztekium ritteri (Rodríguez-Garay and Rubluo, 1992), M. candida (Elias-Rocha et al., 1998), Obregonia denegrii and Coryphantha minima (Malda et al., 1999b). However, optimization of multiplication and rooting media, as well a refinement of the acclimatization transition, is necessary for each species of interest (Hubstenberger et al., 1992). In several studies it has also been demonstrated that in vitro development of cacti plantlets can be extremely rapid in comparison with ex vitro-cultured seedlings. This fact confers an additional advantage to this technology when applied to species of very slow growth (Ault and Blackmon, 1987; George, 1996). For example, Malda et al. (1999a) reported that in vitro-cultured plants of Coryphantha minima grew seven-fold larger than plants cultured under similar ex vitro conditions. This was due to plant growth regulators, high relative humidity and high sugar concentration in the culture media, and an increased photosynthetic rate.

In this work we are reporting systems for the in vitro propagation of Pelecyphora aselliformis and P. strobiliformis, two endangered Mexican species of cacti with extremely slow growth that are difficult to propagate by conventional methods. These systems could become valuable tools for conservation and rational use of these species.

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TABLE 1

EFFECTS OF EXPLANT TYPE, CYTOKININ AND SUCROSE CONCENTRATION ON SHOOT REGENERATION AND PROLIFERATION BY AREOLE ACTIVATION IN PELECYPHORA ASELLIFORMIS

Explant	Growth regulators (μM)	Sucrose $g\,l^{-1}$	Shoots per explant (mean ± SE) First culture cycle (60 d)	Third culture cycle (180 d)[z]
Apical	None	30	1.70 ± 0.24 f	ND
		50	1.33 ± 0.17 f	ND
	2.2 BA	30	7.77 ± 0.76 b	88.27 ± 7.25 b
		50	2.67 ± 0.33 ef	ND
	4.4 BA	30	13.23 ± 1.41 a	122.27 ± 9.05 a
		50	4.36 ± 0.51 cde	ND
	8.8 BA	30	13.70 ± 1.37 a	128.07 ± 9.16 a
		50	3.27 ± 0.32 def	ND
	9.8 2iP	30	5.63 ± 0.60 bcd	ND
		50	3.20 ± 0.34 def	ND
	19.7 2iP	30	6.63 ± 0.58 bc	ND
		50	4.63 ± 0.52 cde	ND
	2.27 TDZ	30	2.33 ± 0.37 ef	ND
		50	2.90 ± 0.40 ef	ND
Transverse	None	30	0.40 ± 0.14 h	ND
		50	0.63 ± 0.23 h	ND
	2.2 BA	30	2.80 ± 0.45 fgh	ND
		50	5.30 ± 0.74 cdef	ND
	4.4 BA	30	7.93 ± 0.69 bc	102.07 ± 4.96 a
		50	7.76 ± 0.51 bcd	ND
	8.8 BA	30	13.03 ± 1.66 a	101.87 ± 5.45 a
		50	6.36 ± 0.75 bcde	ND
	9.8 2iP	30	2.37 ± 0.47 gh	ND
		50	4.67 ± 0.68 efg	ND
	19.7 2iP	30	5.10 ± 0.70 defg	ND
		50	8.33 ± 0.98 b	59.07 ± 6.18 b
	2.27 TDZ	30	2.73 ± 0.85 fgh	ND
		50	0.73 ± 0.27 h	ND

[z] In the third culture cycle we only analyzed the three best treatments for each explant type and species.
Means followed by the same letter within a column do not differ significantly at $P \leq 0.05$.
ND, not determined.

Materials and Methods

Plant material. In vitro-germinated seedlings of Pelecyphora aselliformis and P. strobiliformis were used as tissue sources. Seeds were washed five times with 0.1% Extran (Merck-México, Naucalpan de Juárez, Mexico) in water, then disinfected for 1 min in 70% ethanol, 25 min in 2% sodium hypochlorite, and rinsed four times with sterile distilled water. Seeds were germinated in culture vessels containing MS medium (Murashige and Skoog, 1962) at pH 5.7 with $30\,g\,l^{-1}$ sucrose and solidified with $8\,g\,l^{-1}$ agar. The cultures were maintained for 3–6 mo. on a 16/8-h light/dark cycle under fluorescent light ($54\,\mu mol\,m^{-2}\,s^{-1}$, daylight lamps) at 25 ± 2°C. These same incubation conditions were used in all subsequent experiments. Due to the difficulty in obtaining plant material of Pelecyphora, we had less than 100 seeds of each species (obtained from two different clones of each species). For this reason, plantlets obtained from these seeds were subjected to a preliminary in vitro multiplication cycle with the purpose of obtaining enough plant material for our experiments. For this, the root systems were excised and the shoots were inoculated onto MS basal medium at pH 5.7 containing $30\,g\,l^{-1}$ sucrose, 2.22 μM 6-benzylaminopurine (BA) and solidified with $8\,g\,l^{-1}$ agar. From these initial cultures sufficient biological material was obtained for experiments on axillary shoot proliferation.

Axillary shoot proliferation. The 20–25 mm shoots obtained from the preliminary in vitro multiplication cycle were used as a source of explants for the mass proliferation experiments. This proliferation was carried out by means of axillary bud activation. Two different explant types were tested: apical explants and transverse segments approximately 4 mm wide (shoots without apex cut transversely). The explants were placed into culture jars (baby food jars), capped with polypropylene caps (Magenta Corp., Chicago, IL), containing 30 ml of MS basal media with 30 or $50\,g\,l^{-1}$ sucrose, $10\,g\,l^{-1}$ agar and several treatments with plant growth regulators (see Tables 1 and 2). These treatments were based on their effectiveness with other cacti species (Pérez-Molphe-Balch et al., 1998) and results of previous experiments with these Pelecyphora species (data not shown). We used 25–30 explants of each type per treatment. These experiments were conducted at two independent times for each species. The number of shoots produced in each explant was recorded after 60 d of incubation. Primary explant segments with shoots were subcultured every 60 d on 100 ml of fresh shoot induction medium in polypropylene culture vessels (Phytacon TM) for continued proliferation. Shoot production was evaluated again after three culture cycles (180 d). This was done for the three best treatments for each explant type and species, and 15 primary explants with shoots were used in each treatment.

Shoot elongation. Shoot masses generated in the proliferation experiments were fragmented into four to six pieces and subcultured on the same proliferation medium or transferred to MS basal medium with $30\,g\,l^{-1}$ sucrose and $10\,g\,l^{-1}$ agar or to MS basal medium with $30\,g\,l^{-1}$ sucrose, $10\,g\,l^{-1}$ agar and $3\,g\,l^{-1}$ activated charcoal for shoot elongation. The size of shoots was evaluated after 60 d of incubation on the three tested media.

Rooting of shoots. Shoots were collected from shoot elongation media and used for rooting experiments. The rooting technique consisted of transferring the shoots to MS basal medium with $30\,g\,l^{-1}$ sucrose, $10\,g\,l^{-1}$ agar and four different concentrations of auxins: (1) 2.85 μM indoleacetic acid (IAA); (2) 5.71 μM IAA; (3) 2.46 μM indolebutyric acid (IBA); and (4) 4.90 μM IBA. For this rooting experiment 30 shoots of each species were

TABLE 2

EFFECTS OF EXPLANT TYPE, CYTOKININ AND SUCROSE CONCENTRATION ON SHOOT REGENERATION AND PROLIFERATION BY AREOLE ACTIVATION IN PELECYPHORA STROBILIFORMIS

Explant	Growth regulators (μM)	Sucrose ($g\,l^{-1}$)	Shoots per explant (mean ± SE)	
			First culture cycle (60 d)	Third culture cycle (180 d)[z]
Apical	None	30	1.36 ± 0.13 e	ND
		50	1.20 ± 0.07 e	ND
	2.2 BA	30	2.47 ± 0.27 cde	ND
		50	3.80 ± 0.45 c	ND
	4.4 BA	30	6.43 ± 0.40 b	136.33 ± 12.6 a
		50	6.23 ± 0.66 b	66.13 ± 7.12 b
	8.8 BA	30	10.20 ± 1.05 a	129.0 ± 12.9 a
		50	9.50 ± 0.98 a	87.2 ± 6.11 b
	9.8 2iP	30	2.47 ± 0.38 cde	ND
		50	1.93 ± 0.21 de	ND
	19.7 2iP	30	3.50 ± 0.54 cd	ND
		50	2.00 ± 0.18 cde	ND
	2.27 TDZ	30	2.47 ± 0.30 cde	ND
		50	2.07 ± 0.29 cde	ND
Transverse	None	30	0.26 ± 0.11 e	ND
		50	0.50 ± 0.18 e	ND
	2.2 BA	30	3.10 ± 0.62 de	ND
		50	7.90 ± 0.96 bc	ND
	4.4 BA	30	9.30 ± 0.99 ab	99.87 ± 5.90 a
		50	12.37 ± 1.21 a	119.33 ± 9.66 a
	8.8 BA	30	10.66 ± 1.32 ab	111.93 ± 15.0 a
		50	5.77 ± 0.77 cd	ND
	9.8 2iP	30	2.26 ± 0.43 e	ND
		50	2.90 ± 0.46 de	ND
	19.7 2iP	30	3.37 ± 0.52 de	ND
		50	5.63 ± 1.88 cd	ND
	2.27 TDZ	30	1.63 ± 0.48 e	ND
		50	2.40 ± 0.50 e	ND

[z] In the third culture cycle we only analyzed the three best treatments for each explant type and species.
Means followed by the same letter within a column do not differ significantly at $P \leq 0.05$.
ND, not determined.

used per treatment. The production of roots was evaluated 6 wk after initiating the experiment. Shoots that developed roots ≥15 mm in length were scored positively. The experiments were conducted at two independent times for each species.

Acclimatization and transfer of plantlets to soil. The rooted plants were transplanted to pots containing a mix of ground-sand and soil (1:1), covered with plastic bags for 2–3 wk to prevent desiccation and allowed to acclimatize before being transferred to the greenhouse. Survival percentages were determined 16 wk after transplantation.

Data analysis. Data were analyzed using ANOVA and means were compared by Tukey-Kramer multiple range test ($P \leq 0.05$).

Results and Discussion

Due to limited seed availability and low germination rate (approximately 50% in both species), it was necessary to carry out a preliminary in vitro proliferation cycle to obtain the necessary plant material for further experiments. This was done by transferring the plantlets obtained from the seeds to a medium containing 2.22 μM BA. In this initial proliferation cycle an average of 6.5 and 3.8 new shoots per plantlet were obtained after 60 d of incubation for P. aselliformis and P. strobiliformis, respectively. These shoots were used as a source of explants for the proliferation experiments. In these experiments, the explants of the two species exhibited shoot production from the areoles after 60 d of incubation in all treatments containing cytokinins. In the range of concentrations used, BA was more effective than N^6-[2-isopentenyl]adenine (2iP) and thidiazuron (TDZ) for activation of axillary buds (Tables 1 and 2). The positive effect of BA on the capacity to induce plant regeneration in members of the Cactaceae has been reported previously (Escobar et al., 1986; Martínez-Vázquez and Rubluo, 1989), although there are also reports that indicate that 2iP is a more appropriate cytokinin for some species (Hubstenberger et al., 1992).

Besides cytokinins, we tested two sucrose concentrations, 30 and $50\,g\,l^{-1}$ (Tables 1 and 2). However, there was no consistent difference between these two sucrose concentrations with regard to shoot production. These results contrast with those of Escobar et al. (1986), who reported that $50\,g\,l^{-1}$ was the optimal sucrose concentration for shoot formation in Opuntia amyclaea. We observed the highest proliferation rate (13.7 shoots per explant) in P. aselliformis with 8.8 μM BA and $30\,g\,l^{-1}$ sucrose using apical explants. However, there was no significant difference when 4.4 μM BA was used (13.2 shoots per explant) (Table 1). The 4.4 μM BA treatment would be more convenient to use for proliferation to avoid the adverse effect of the use of higher levels of BA, such as hyperhydricity, callus formation, somaclonal variation, and decrease

FIG. 1. In vitro propagation of Pelecyphora aselliformis and P. strobiliformis. a, Shoot proliferation in apical and transverse explants of P. aselliformis obtained with 8.8 μM BA after 60 d of culture initiation. b, Shoot proliferation in apical and transverse explants of P. strobiliformis obtained with 8.8 μM BA after 60 d of culture initiation. c, Elongated shoots of P. aselliformis after 60 d in medium with 3 g l^{-1} activated charcoal. d, Elongated shoots of P. strobiliformis after 60 d in medium with 3 g l^{-1} activated charcoal. e, Shoots of P. strobiliformis rooted in medium with 4.90 μM IBA. f, In vitro-generated plants of P. aselliformis growing in soil.

TABLE 3

EVALUATION OF THE INFLUENCE OF THREE CULTURE MEDIA ON SHOOT ELONGATION IN PELECYPHORA ASELLIFORMIS AND P. STROBILIFORMIS

Species	Shoot elongation treatment	Length of shoots in mm (mean ± SE)
P. aselliformis	Proliferation medium (MS with 8.8 μM BA)	10 ± 0.25 b
	MS basal medium	10 ± 0.24 b
	MS basal medium with 3 g l^{-1} activated charcoal	12 ± 0.29 a
P. strobiliformis	Proliferation medium (MS with 8.8 μM BA)	10 ± 0.20 b
	MS basal medium	11 ± 0.21 b
	MS basal medium with 3 g l^{-1} activated charcoal	15 ± 0.37 a

Means followed by the same letter within each species do not differ significantly at $P \leq 0.05$.

of rooting efficiency. In P. strobiliformis the highest proliferation rate, 12.4 shoots per explant, was observed using 8.8 μM BA and 50 g l^{-1} sucrose, but using shoot transverse segments as explants. In this case, the use of lower levels of BA reduced significantly the yield of buds (Table 2).

Although we did not find previous reports on micropropagation of the species reported here, their proliferation rates are superior to those reported for other cacti such as Ferocactus acanthodes (7.9 shoots per explant) (Ault and Blackmon, 1987), Mediocactus coccineus (7.8 shoots per explant) (Infante, 1992), or Mammillaria candida (seven shoots per explant) (Elias-Rocha et al., 1998). In our case, all new shoots were originated from the axillary buds in the areoles. Micropropagated cacti regenerated from axillary buds are considered to be genetically stable (Machado and Prioli, 1996). Maintaining genetic stability in regenerated plants is essential for endangered species conservation. Other regeneration systems, such as those from callus, have the potential problem of somaclonal variation (Oliveira et al., 1995).

In the case of transverse explants, the shoots generated were homogeneous in their size and differentiation stage. When apical explants were used, a dominant shoot of larger size arose surrounded by some small shoots. The dominant shoot arises from the elongation of the inoculated apex, and the small shoots appear at its base (Fig. 1a, b). When the primary explants with shoots were kept in the proliferation media with cytokinins (with fresh medium upon subcultures and transfer to larger culture vessels), continuous proliferation occurred. This consisted of the appearance of new shoots formed from the areoles of primary shoots.

To investigate how many new shoots could be obtained from these continuous proliferation systems, we evaluated the yield of shoots from the original explants in the three best proliferation media for each explant type and species after three culture cycles (180 d). Under these conditions, P. aselliformis and P. strobiliformis can yield up to 128.1 and 136.3 new shoots starting from a single explant, respectively (Tables 1 and 2). Although such continuous proliferation systems were productive, the shoots generated were small and in preliminary trials their rooting was difficult (data not shown). For this reason, elongation of these shoots in other media was tested. A comparison of shoot elongation in proliferation medium, medium without growth regulators and medium with activated charcoal for both species is presented in Table 3. In both species, the best response was obtained in medium containing activated charcoal (Table 3), where the most vigorous shoots were produced (Fig. 1c, d). These shoots also showed higher rooting efficiency than the original shoots. It has been demonstrated in other plants that shoot elongation and maturation in media with activated charcoal reduces endogenous levels of BA; these levels can remain high after the proliferation stage and it is known that reduction of endogenous BA levels increases rooting (Hemphill et al., 1998).

In the two species, rooting was achieved in all four auxin treatments tested, without significant differences between them. On average, rooting efficiency was 89% for P. aselliformis and 87% for P. strobiliformis. The in vitro-generated roots were vigorous (Fig. 1e) and the survival of the plants once transferred to soil was 88% on average (Fig. 1f).

Hyperhydration of the tissues is a serious problem for in vitro culture of cacti (Elias-Rocha et al., 1998). This physiological disorder is due to the physical and chemical conditions of in vitro culture; i.e. high humidity, excess of carbohydrates and minerals, high levels of plant growth regulators and low light intensity (Ziv, 1991). In this work, hyperhydration was present in less than 5% of total shoots. This low percentage was due to two factors: (1) the use of high concentrations of agar (10 g l^{-1}), which diminishes water availability in the medium, and (2) by using low concentrations of plant growth regulators in the proliferation media. These conditions reduce hyperhydration in cactus tissue culture (Elias-Rocha et al., 1998; Pérez-Molphe-Balch et al., 1998).

In conclusion, efficient protocols for mass propagation of P. aselliformis and P. strobiliformis were established. These protocols are considerably more efficient than traditional propagation methods and can be valuable tools for: (1) the in vitro conservation of these species; (2) production of plants for their distribution to the scientific community or commercialization so that further collection of wild plants is unnecessary; (3) the massive production of plants for the repopulation of damaged natural areas (in this case, micropropagated plants from several different clones could be used to avoid the risks of monoculture); and (4) phytochemical studies in these species.

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